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TT Virus Infection in Patients With Chronic Hepatitis B or C: Influence on Clinical, Histological and Virological Features

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Concomitant infection with TT virus and hepatitis B virus (HBV) or hepatitis C virus (HCV) is common. However, the effect of TTV infection on chronic hepatitis B or C is unknown. The prevalence of TTV infection, the effect of TTV infection on the clinical, histological and virological features of patients with chronic hepatitis B or C, and the influence of TTV infection on the HCV response to interferon alfa therapy were studied. A total of 100 asymptomatic hepatitis B surface antigen carriers, 220 patients with HBV-related chronic liver diseases, and 110 patients with chronic hepatitis C treated with interferon alfa (3 million units subcutaneously three times a week for 24 weeks) were enrolled. Serum HCV RNA and serum TTV DNA were detected by the polymerase chain reaction (PCR). Serum HBV DNA and serum HCV RNA level were quantified by branched DNA assays. Infection with TTV was detected in 21.5% of HBV carriers and 37% of HCV carriers. TTV infection had little effect on the clinicopathological course of chronic HBV infection. In chronic hepatitis C, clinical features, histological severity, serum HCV RNA levels, and the response to interferon alfa therapy did not differ between those with and without TTV infection. The loss of serum TTV DNA did not correlate with the biochemical response as did in the loss of serum HCV RNA. In conclusion, TTV infection is found frequently in patients with chronic hepatitis B or C in Taiwan; however, coinfection with TTV does not affect the clinicopathological course of chronic hepatitis B or C and the response to interferon alfa therapy. ***J. Med. Virol. 60:387–392, 2000.***

KEY WORDS: chronic hepatitis B; chronic hepatitis C; chronic liver disease; hepatocellular carcinoma; liver cirrhosis; TT virus

INTRODUCTION

Chronic liver disease and hepatocellular carcinoma (HCC) are endemic in Taiwan, and most are caused by HBV or HCV infection [Chen et al., 1987; 1990]. However, there still remains a proportion of hepatitis cases with undefined etiology, suggestive of the existence of additional causative agents [Alter 1994; Kao et al., 1996b]. Recently, a DNA virus was isolated from a patient with posttransfusion hepatitis of unknown etiology and designated TT virus (TTV) for the initials of the index patient [Nishizawa et al., 1997]. In addition, TTV genomes were detected in patients with cryptogenic posttransfusion hepatitis viraemia coincided with modest increases of serum alanine aminotransferase (ALT) levels [Okamoto et al., 1998]. A recent study showed that TTV genome is circular and negative stranded, and comprises 3,852 bases with a particle size of 30–50 nm, suggesting TTV is similar to the *Circoviridae* [Mushahwar et al., 1999]. In Japan, TTV DNA was detected in 12% of healthy blood donors, 47% of patients with fulminant non-A~E hepatitis and 46% of patients with chronic liver diseases of unknown etiology [Okamoto et al., 1998], suggesting that TTV may be the cause of some cryptogenic liver diseases. However, the clinical significance of infection with TTV alone or in combination with other hepatitis viruses has been questioned [Charlton et al., 1998; Naoumov et

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al., 1998; Simmonds et al., 1998; Prati et al., 1999]. Taking advantage of the common chronic HBV and HCV infections in Taiwan, the prevalence of TTV infection and the possible role of TTV coinfection on the clinical, virological and histological features of patients with chronic hepatitis B or C were studied. In addition, the response of HCV and TTV to interferon-alfa treatment was evaluated.

MATERIALS AND METHODS

Patients

Serum samples were studied retrospectively from 490 patients with long-term follow-up at the gastroenterological clinics of the National Taiwan University Hospital. These included (i) 100 asymptomatic hepatitis B surface antigen (HBsAg) carriers (54 men, 46 women; mean age, 30 ± 7 years) with normal serum ALT level for at least one year, and 220 histologically verified HBsAg-positive chronic liver disease and HCC patients (173 men, 47 women; mean age, 45 ± 12 years); (ii) 170 histologically verified HCV-related chronic liver disease and HCC patients (102 men, 68 women; mean age, 50 ± 10 years). 110 of the patients with chronic hepatitis C (71 men, 39 women; mean age, 45 ± 12 years) had received previously 3 million units of interferon alfa (IFN) thrice weekly for 24 weeks. The presence of HCV RNA and TTV DNA in the serum was determined before initiation of IFN therapy; at the end therapy; and 24 weeks after the therapy was discontinued. The response to IFN was classified into two patterns according to the serum ALT level. Patients who had normalized serum ALT levels (≤ 40 U/L) at the end of therapy and during the follow-up period was considered to have a sustained biochemical response. Non-sustained response was defined as serum ALT levels that could not be normalized either at the end of therapy or during follow-up period. The diagnosis of chronic liver disease was based on clinical and pathological grounds accepted generally including chronic persistent hepatitis (CPH), chronic active hepatitis (CAH), liver cirrhosis (LC) and HCC. Those with dual infections by HBV and HCV were excluded. An HBV carrier was defined by the presence of HBsAg, and HCV carriers by both second-generation anti-HCV and HCV RNA positivity for at least six months. All the enrolled patients had no markers suggestive of autoimmune hepatitis including antinuclear antibodies, antimitochondrial antibodies and anti-smooth muscle antibodies. None had a history of alcoholism (> 50 gm/day), injection drug abuse, homosexuality, or hepatotoxic drug intake. Metabolic liver disease including haemochromatosis, Wilson's disease or α-1 anti-trypsin deficiency was excluded by clinical and laboratory data. In addition, 100 healthy adults who were at no risk for hepatitis and were with normal serum ALT level and without serological markers of current hepatitis viral infection were used as a control group. Serum samples taken from each subject were stored at −70°C until use.

Serological Markers

HBsAg and anti-HCV were tested with available commercial kits (Ausria II and HCV EIA II, Abbott Laboratories, North Chicago, IL).

Detection of HCV RNA and Genotyping of HCV

Serum HCV RNA was assayed by reverse transcription-polymerase chain reaction (RT-PCR) with primers from the most conserved 5′ untranslating region of the viral genome [Kao et al., 1992], and identification of HCV genotype by type-specific primers as previously described [Kao et al., 1995]. To avoid false-positive results, the methods described by Kwok and Higuchi [1989] to prevent cross contamination were applied.

Quantitation of HBV DNA and HCV RNA

Serum HBV DNA level was quantified by a branched DNA (bDNA) signal amplification assay (Hepatitis B Viral DNA, Chiron, Emeryville, CA) as described previously [Kao et al., 1998]. The HBV DNA quantification range of the bDNA assay is 2.5 ~ 17,700 pg/mL. Serum HCV RNA level was determined by using a second generation bDNA signal amplification assay (Quantiplex-HCV, version 2.0; Chiron) with a detection limit of 0.2 MEq/mL according to the manufacturer's instructions.

Detection of TTV DNA

The presence of TTV DNA was assayed by nested PCR with primer pairs from the open reading frame (ORF)-1 of the viral genome [Kao et al., 1999]. Briefly, total DNA was extracted from 100 μL serum using QIAamp Blood kit (QIAGEN Ltd, Crawley, UK) and resuspended in 50 μL elution buffer. For the first stage PCR, a 25 μl of reaction mixture containing 2 μl of the DNA sample, 1x PCR buffer (10 mM tris-HCl pH 9.0, 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin and 0.1% Triton X-100), 10 mM of each dNTP, 100 ng of each outer primer (outer sense: T-1s 5′-ACAGACAGAGGAGAAGGCAACATG-3′; outer antisense: T-2a 5′-CTACCTCCTGGCATTTTACC-3′) and 1 unit of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. Each cycle entailed denaturation at 95°C for 60 s, primer annealing at 55°C for 30 s and extension at 72°C for 60 s with a final extension step at 72°C for 7 min. After the first amplification, 1 μl of the PCR products was reamplified for another 30 cycles with 100 ng of each inner primer (inner sense: T-3s 5′-GCAACATGTTATGGATAGACTGG-3′; inner antisense: T-4a 5′-CTGGCATTTTACCATTTCCAAAGTT-3′). The second round of PCR was done in the same manner as the first round giving a 272 bp amplification product. The amplified products were separated in 3% agarose gel electrophoresis and stained by ethidium bromide. Nucleotide sequences of selected amplified products were directly determined by using fluorescence labelled primers with a 373A Sequencer (Applied Biosystems, Foster City, CA) to verify the specificity.

TABLE I. Prevalence of Serum TT Virus (TTV) DNA in 320 Hepatitis B Surface Antigen Carriers With Different Liver Diseases

Diagnosis	No. studied	Sex (M/F)	Age (yr)	TTV DNA	
				No. positive	%
Asymptomatic carrier	100	54/46	30 ± 7	12	12*
CPH	50	39/11	35 ± 8	10	20
CAH	50	42/8	41 ± 9	8	16
Liver cirrhosis	50	40/10	46 ± 11	10	20
HCC	70	52/18	55 ± 13	29	41*
Total	320			69	21.5

*$P < 0.001$.

Statistical Analysis

Data were analyzed by Chi-square test with Yates' correction or Student's t test where appropriate. A P value of less than 0.05 was considered significant.

RESULTS

Of 430 patients with chronic hepatitis viral infections, the overall prevalence of TTV viraemia was 21.5% and 37% in HBV and HCV carriers, respectively, which was significantly higher than that in healthy adults (10%, Kao et al., 1999, $P < 0.02$ and < 0.001, respectively).

In 320 HBV carriers with various liver diseases (Table I), the prevalence of TTV DNA ranged from 12% to 20% among asymptomatic carriers, patients with CPH, CAH and liver cirrhosis. However, the prevalence was significantly higher in those with HCC than in asymptomatic carriers (41% vs. 12%, $P < 0.001$). Cases of cirrhosis with HBV and TTV coinfection had a similar proportion of transfusion history to those with HBV infection alone (20% vs. 17%). The average age of HCC patients with both HBsAg and TTV DNA was comparable to that of patients with HBsAg alone (52 ± 13 vs. 56 ± 12 years, $P = 0.2$).

No statistically significant difference was found in gender distribution, mean age, frequency of transfusion history, mean peak serum ALT level, and histological severity when the 18 patients with HBV and TTV coinfection were compared with the 82 patients with HBV infection alone (Table II). However, patients with both HBV and TTV infection tended to have a lower mean serum HBV DNA level than those with HBV infection alone ($P = 0.066$).

In 170 patients with HCV-related chronic liver diseases, the prevalence of TTV DNA decreased with the severity of chronic liver disease (Table III). It was highest in patients with CPH (41%) and was lowest in patients with HCC (17%, $P = 0.034$). Cases of liver cirrhosis with HCV and TTV coinfection had a higher frequency of past transfusion than those with HCV infection alone (67% vs. 21%, P = 0.09). The mean age of HCC patients possessing both HCV RNA and TTV DNA was significantly lower than that of patients possessing HCV RNA alone (56 ± 5 vs. 64 ± 8 years, $P = 0.04$).

Among 110 patients with chronic hepatitis C, HCV genotypes 1b, 2a, 2b and mixed infection were found in 71, 24, 9, and 6, respectively. When these patients were stratified by the presence or absence of TTV DNA, there was no significant difference in clinicopathological features including gender distribution, mean age, percentage of transfusion history, mean peak serum ALT level, histological severity, and distribution of HCV genotypes (Table IV). Although patients with HCV and TTV coinfection had a lower mean serum HCV RNA level than those with HCV infection alone, the difference was not statistically significant ($P = 0.5$). In addition, the biochemical response to interferon alfa therapy did not differ between patients with and without TTV infection (Table III). The sustained response rate was 20% and 17% in patients with TTV DNA and in those without TTV DNA, respectively.

Of the 41 patients with HCV and TTV coinfection before initiation of interferon therapy, 29 (70%) lost serum TTV DNA at the end of therapy as determined by PCR, and 17 (41%) remained serum TTV DNA nega-

TABLE II. Demographic and Clinical Data of 100 Cases of Chronic Hepatitis B With and Without TT Virus (TTV) DNA

Characteristics	Serum TTV DNA	
	Positive	Negative
No. of cases	18	82
Sex (M/F)	13/5	68/14
Age (yr)	37 ± 9	38 ± 10
Transfusion history	1 (6%)	3 (4%)
Peak ALT level (U/L)	183 ± 77	166 ± 52
Histology		
CPH	10 (56%)	40 (49%)
CAH	8 (44%)	42 (51%)
Serum HBV titer (pg/mL)	2986 ± 4703*	5860 ± 6176*

*$P = 0.066$.

TABLE III. Prevalence of Serum TT Virus (TTV) DNA in 170 Hepatitis C Virus Carriers With Different Liver Diseases

Diagnosis	No. studied	Sex (M/F)	Age (yr)	TTV DNA	
				No. positive	%
CPH	79	51/28	43 ± 12	32	41*
CAH	31	20/11	50 ± 9	9	29
Liver cirrhosis	30	13/17	56 ± 9	6	20
HCC	30	18/12	63 ± 8	5	17*
Total	170			52	30.6

*$P = 0.034$.

TABLE IV. Demographic and Clinical Data of 110 Cases With Chronic Hepatitis C With and Without TT Virus (TTV) DNA

Characteristics	Serum TTV DNA	
	Positive	Negative
No. of cases	41	69
Sex (M/F)	30/11	41/28
Age (yr)	44 ± 11	46 ± 13
Transfusion history	12 (29%)	17 (25%)
Peak ALT level (U/L)	137 ± 102	123 ± 62
Histology		
CPH	32 (78%)	47 (68%)
CAH	9 (22%)	22 (32%)
HCV genotype		
1b	23 (56%)	48 (70%)
2a	11 (27%)	13 (19%)
2b	6 (15%)	3 (4%)
Mixed	1 (2%)	5 (7%)
Serum HCV titer (MEq/mL)	1.3 ± 4.3	1.8 ± 3.6
Interferon response		
Sustained responder	8 (20%)	12 (17%)
Nonsustained responder	33 (80%)	57 (83%)

tive after stopping therapy for 6 months. However, no correlation was found between the sustained biochemical response and the loss of serum TTV DNA. Six months after stopping therapy, serum TTV DNA was not detectable in 4 of 8 patients (50%) who had sustained biochemical responses compared to 13 of 33 patients (39%) who did not respond ($P = 0.9$). On the contrary, a correlation was seen between sustained biochemical response and sustained loss of serum HCV RNA (Fig. 1).

DISCUSSION

Although a transfusion-transmissible flavi-like RNA virus, GB virus-C (GBV-C)/hepatitis G virus (HGV) [Simons et al., 1995; Linnen et al., 1996], has been claimed to be associated with fulminant and chronic hepatitis in initial reports [Yoshiba et al., 1995], most of the subsequent studies indicated that GBV-C does not cause liver disease as classical hepatitis viruses [Wang et al., 1996; Kao et al., 1996a; 1997; Alter et al., 1997]. And thus, the search of a new hepatitis virus goes on. A novel virus in association with hepatitis flares was discovered from a patient with posttransfusion hepatitis of unknown etiology [Nishizawa et al., 1997]. The virus has been characterized recently and is similar to the Circoviridae [Mushahwar et al., 1999]. TTV may replicate in liver cells, because its DNA is detected in the liver in titers 10 to 100 times higher than in the corresponding serum from some patients with chronic non-A~E hepatitis [Okamoto et al., 1998]. These findings render TTV an attractive candidate virus for causing liver disease. In contrast, subsequent studies have challenged the initial observations, and an overwhelming body of evidence has indicated that TTV infection is not associated with liver damage [Charlton et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998; Prati et al., 1999].

Although reliable serological assays are not available, previous studies based on PCR procedures to detect TTV DNA in serum samples from different populations have shown that the virus is transfusion-transmissible, distributed globally and can induce persistent viremia in humans [Okamoto et al., 1998; Simmonds et al., 1998; Prati et al., 1999]. In general, TTV is common in populations at risk of infection with blood-borne viruses, such as hemophiliacs or patients on maintenance hemodialysis, and abusers of intravenous drugs [Hohne et al., 1998; Okamoto et al., 1998; Poovorawan et al., 1998; Takayama et al., 1999]. However, transmission modes other than parenteral routes have been suggested due to the high prevalence of TTV viraemia in healthy population [Zuckerman 1999].

Many hepatitis viruses share the same modes of transmission, thus multiple viral infection may occur in a given patient [Pontisso et al., 1993]. Coinfection of TTV has been observed frequently in patients with chronic hepatitis B and C [Naoumov et al., 1998]. Taking advantage of the extremely common chronic HBV and HCV infections in Taiwan, we investigated the presence of simultaneous TTV infection in HBV and HCV carriers. In the present study, we consistently found a significantly higher overall prevalence of TTV infection in patients with chronic hepatitis B (22%) or C (37%) than in healthy adults (10%) [Kao et al., 1999], implying that HBV, HCV and TTV may share common modes of transmission. By contrast, the prevalence in asymptomatic HBsAg carriers (12%) was similar to that in the general population (Table I). These findings are not unanticipated because most HBsAg carriers in Taiwan contracted HBV infection during their perinatal periods or early childhood [Chen 1987], and superinfection of TTV may occur thereafter.

Although active infection of TTV is observed frequently in patients with chronic hepatitis B and C, the interaction between TTV and HBV or HCV in such patients remains unclear. The data showed a relatively constant TTV DNA prevalence (16~20%) among patients with HBV-related CPH, CAH, and cirrhosis (Table I), and a significantly increased prevalence in patients with HCC compared to asymptomatic HBV carriers (41% vs. 12%, $P < 0.001$), implying an association between TTV and HCC. However, such a result does not necessarily represent a causative role of TTV in the development of HCC among HBV carriers. The comparable mean age between HCC patients with both HBsAg and TTV DNA and those with HBsAg alone (52 ± 13 vs. 56 ± 12 years, $P = 0.2$) supported this interpretation. Accordingly, the higher prevalence of TTV infection in HCC patients may simply reflect greater chances of parenteral and non-parenteral exposure to TTV during their illness than was experienced by asymptomatic HBV carriers.

Among patients with HBV-related CPH and CAH, the clinical and histological features were comparable between those with and without TTV coinfection (Table II). Previous studies have demonstrated that HCV superinfection could exert a suppressive or inhibitory ef-

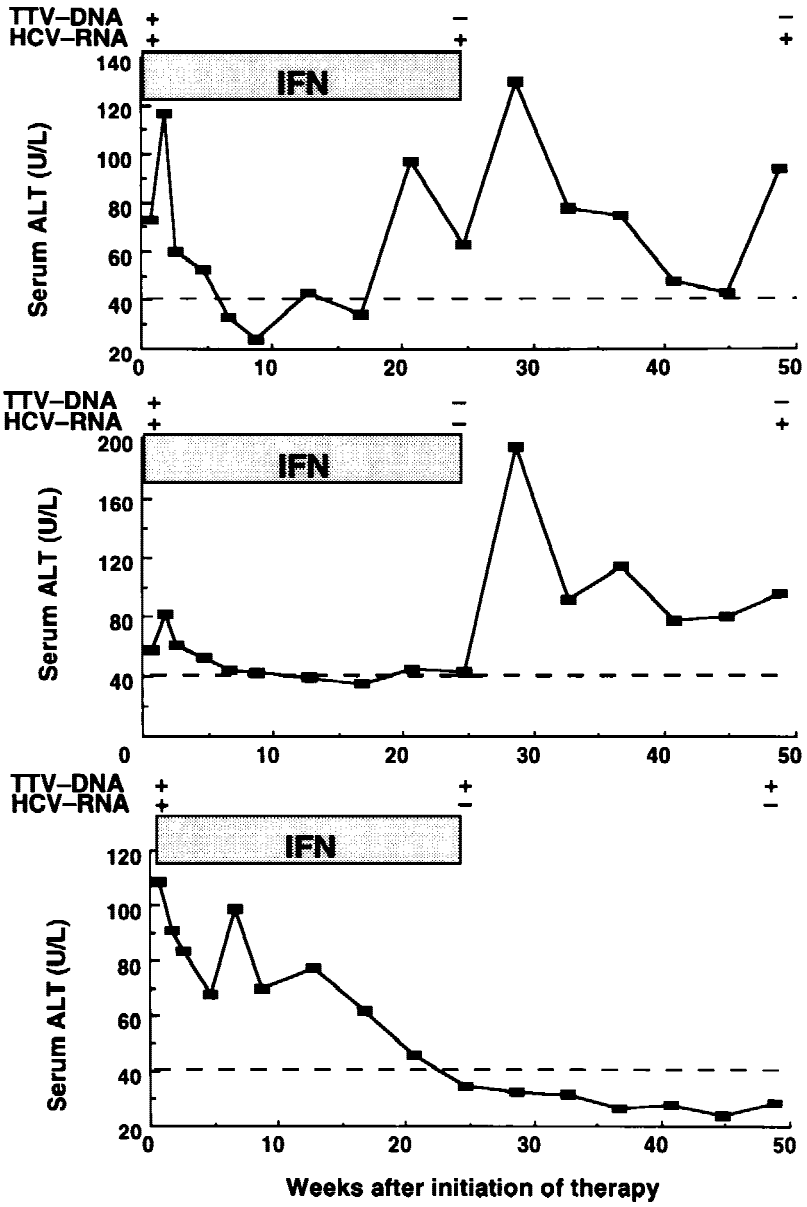

Fig. 1. Changes of serum ALT levels, HCV RNA, and TTV DNA in 3 representative patients having chronic hepatitis C coinfected with TTV and receiving treatment with 3 million units of IFN 3 times weekly for 24 weeks. A correlation was seen between serum ALT levels and serum HCV RNA response, however, such correlation was not found for serum TTV DNA.

fect on the replication of preexisting HBV [Pontisso et al., 1993]. Whether this phenomenon also holds true for TTV infection is unknown. In this study, there was a tendency of lower serum HBV DNA levels in patients with both HBV and TTV infection than those with HBV infection alone (2986 ± 4703 pg/mL vs. 5860 ± 6175 pg/mL, $P = 0.066$). Taken together, these observations suggested that TTV has no influence on the clinicopathological course of chronic HBV infection.

In contrast to the patients with HBV infection, in those with chronic HCV infection, the prevalence of serum TTV DNA decreased from 41% steadily to 17% among patients with CPH, CAH, LC and HCC (Table III). The lower TT viremia in HCV-induced end-stage liver disease is not due to the liver status *per se*, as this did not happen in those caused by HBV. This steady decrease of viremia from chronic hepatitis to HCC was not seen in GBV-C either (data not shown). And thus, there might be specific interactions between TTV and HCV. Perhaps HCV may suppress the replication of TTV in a given patient as in the case of HCV superinfection in HBV carriage [Pontisso et al., 1993].

In chronic hepatitis C, TTV infection did not influence the severity of liver disease (Table IV). Serum ALT levels and histological severity in patients with TTV coinfection did not differ from those in patients with HCV infection alone. This result is consistent with the view that liver lesions are related to HCV rather than to TTV infection, leading to the suspicion that TTV has little or no pathogenecity. This is further confirmed by the absence of correlation between serum TTV DNA positivity and normalization of serum ALT

level during and after interferon alfa therapy (Fig. 1). In contrast, serum HCV RNA positivity was associated closely with the biochemical response, indicating the hepatitis activity in these patients was related primarily to HCV infection (Fig. 1).

The influence of TTV coinfection on the response of HCV to interferon alfa therapy is unknown. Our results showed that the sustained response rate to interferon alfa therapy was similar between chronic hepatitis C patients with and without TTV infection, suggesting TTV does not interfere the HCV response to interferon alfa. In addition, the sustained virological response rate of TTV to interferon alfa remains virtually unexplored. The data showed that 29 of 41 chronic hepatitis C patients (70%) with TTV coinfection lost serum TTV DNA at the end of interferon alfa therapy, and 17 (41%) had a sustained virological remission after stopping therapy for six months. These facts indicated that TTV is actually an interferon-sensitive virus, and if proven to be pathogenic, further studies to define better the optimal dose and duration of interferon treatment are then required.

In summary, these results showed that in Taiwan TTV infection is frequent in patients with chronic hepatitis B or C; however, coinfection of TTV has no effect on the clinicopathological status of chronic hepatitis B or C and the therapeutic response to interferon alfa. The natural course of chronic TTV infection and whether its coinfection affects the natural history of chronic type B or type C hepatitis await long-term longitudinal studies.

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